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(71) Applicants:
- **SOREMARTEC S.A.**
  6700 Arlon-Schoppach (BE)
  Designated Contracting States:
  BE ES FR GB NL
- **Ferrero S.p.A.**
  I-12051 Alba (Cuneo) (IT)
  Designated Contracting States:
  IT

(72) Inventors:
- **Dani, Maria**
  81020 Sala di Caserta (IT)
- **Catello, Sergio**
  80129 Napoli (IT)

(74) Representative: **Rambelli, Paolo et al**
c/o JACOBACCI & PERANI S.p.A.
Corso Regio Parco, 27
10152 Torino (IT)

(54) **Isolation and sequencing of the hazel FAd2-N gene**

(57) The invention relates to the isolation from hazel (*Corylus avellana L.*) of the FAD2-N gene coding for the Δ12 desaturase enzyme of the microsomal fraction and, in particular, provides the nucleotide sequence and the deduced amino-acid sequence of the gene and provides for its use as a probe for the isolation of other plant desaturases. It also relates to the use of this gene for altering the desaturase levels and consequently the fatty-acid composition of the plant.

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Description

The present invention relates to the isolation from hazel (*Corylus avellana L.*) of the FAD2-N gene which codes for the Δ12 desaturase enzyme of the microsomal fraction.

More particularly, the invention relates to the nucleotide sequence, to the derived amino-acid sequence of the gene, and to its use as a probe for the isolation of other plant desaturases. It also relates to the use of this gene for altering the desaturase levels, and consequently the fatty-acid composition of the plant.

Alteration of the fatty-acid composition may have various applications in the industrial field. One of the greatest problems with hazelnuts is that they become rancid by oxidation. This is due to the auto-oxidation of unsaturated lipids with the consequent formation of volatile substances with a rancid odour which cannot easily be eliminated by the usual preservation systems. Amongst the possible strategies for reducing the tendency to become rancid, the best seems to be that of reducing the degree of unsaturation of the fatty acids present in the kernel oil, since susceptibility to auto-oxidation is positively correlated with this parameter. In fact, the rate of peroxide formation is correlated with the number of C=C double bonds in the fatty acids. The rate of auto-oxidation of the fatty acids in comparison with the oleate (18:1) is about 30 times greater in the linoleate (18:2) and 80 times greater in the linolenate (18:3). Moreover, the volatile substances resulting from the degradation of the linoleate and of the linolenate have a lower threshold of perception than those derived from the oleate. A reduction in linoleic acid should reduce the availability of substrates for lipoxygenase, reduce the loss of vitamin E during preservation, and reduce the production of volatile substances such as hexanals.

In the angiosperms, most of the synthesis of polyunsaturated lipids takes place by means of a single enzyme, that is, Δ12 (or ω6) desaturase (18:1 desaturase), of the endoplasmic reticulum, although there is an 18:1 chloroplast desaturase in the leaves of some plants. Moreover, this enzyme is responsible for more than 90% of the synthesis of polyunsaturated fatty acids in non-photosynthetic tissues such as, for example, in the kernels. The conversion of oleic acid (18:1) to linoleic acid (18:2) thus takes place by means of Δ12 desaturase, and from linoleic acid to linolenic acid (18:3) by means of Δ15 (or ω3) desaturase.

It has been shown with mutants of *Arabidopsis* that the FAD2 locus contains a gene which codes for the oleate desaturase enzyme of the endoplasmic reticulum (Okuley et al, 1994, The Plant Cell 6, 147-158). The FAD2 gene was in fact able to complement mutants of *Acabidopsis* which were deficient in desaturase activity of the endoplasmic reticulum. The gene coding for the same enzyme in soya has also recently been isolated and sequenced (Heppard et al, 1995, Plant Physiol., in press).

A reduction in the Δ12 desaturase levels should therefore lead to a reduction in the linoleic acid content and, as a secondary effect, probably also to a reduction in linolenic acid. In hazelnuts the percentage of linoleic acid varies from 5 to 15%; the percentage of linolenic acid is from 0.1 to 0.2%. A reduction in these fatty acids should therefore be useful in the preservation of hazelnuts. There is therefore clearly a need to isolate the gene which codes for the Δ12 desaturase of the endoplasmic reticulum. The sequence of the gene could thus be used for gene inactivation in hazelnut kernels. This inactivation could be carried out either by the antisense technique (Smith et al. (1988) Nature 334, 724-726) or by the "transwitch" technique (Flavell (1994) Proc. Natl. Acad. Sci. USA 91, 3490-3496). In the antisense technique, the hazel would have to be transformed by the entire FAD2-N gene or by portions thereof, inserted in the opposite direction to the regulating sequences. In the "transwitch" technique, the hazel would have to be transformed by an identical copy of the FAD2-N gene.

The subjects of the present invention are defined by the following claims.

Embodiments of the present invention will now be described with reference to the following drawings, in which:

Figure 1 shows the restriction map of the N2 genome clone,

Figure 2 shows the nucleotide sequence of the hazel FAD2-N gene; the amino-acid sequence of the coding portion is also shown;

Figure 3 shows the nucleotide sequence of the "I" clone of cDNA,

Figure 4 shows a comparison between the nucleotide sequences of the "I" and "N2" clones,

Figure 5 shows a comparison between the amino-acids of the "N2" gene and Δ12 desaturases of *Arabidopsis* and of soya,

Figure 6 shows the homology between hazel Δ12 desaturase and various desaturases of other plants both plastid and of the endoplasmic reticulum,

Figure 7 shows the expression of the N2 gene in various varieties of hazel both in the leaves and in the kernels.

Isolation and cloning of the FAD2 gene of *Arabidopsis thaliana* for use as a probe

In order to isolate the gene which codes for hazel Δ12 desaturase enzyme, it was necessary to use the FAD2 gene of *Arabidopsis* as a probe.

In order to isolate the *Arabidopsis gene*, two oligonucleotides were used as "primers" for the amplification of the sequences included between the start and the end of the gene. The oligonucleotides used were NOCC1 (CTGAATTC-CAGGTGGAAGAATGCC) which contains the Eco RI restriction site and the sequences corresponding to the portion between bases 100 and 116 of the gene (Okuley J. et al, 1994, The Plant Cell 6, 147-158) and NOCC4 (AGGAATTC-GACAATTTCTTCACCATCATGC) which contains the restriction site of the Eco RI enzyme and the sequences complementary to the portion between base 1245 and base 1266. The amplification reaction was as follows: 12.8µl H_2O, 2.5µl 10 x PCR buffer (Perkin Elmer), 2.5µl *Arabidopsis* genome DNA(10 ng/l), 1µl dNTP, each 2.5mM, 2µl 25mM $MgCl_2$, 1µl NOCC1 oligonucleotide (50ng/µl), 1µl NOCC4 oligonucleotide (50ng/µl) 0.2µl Taq I DNA polymerase (Perkin Elmer) (5U/µl). The mixture thus prepared was subjected to 1 denaturing cycle for 1 minute at 94°C and to 40 cycles composed as follows: 30 seconds at 94°C, 1 minute at 52°C, 2 minutes at 72°C. The amplification products were separated on 1% agarose gel in TAE buffer (0.04M Tris-acetate, 0.002M EDTA) and stained with ethidium bromide at a concentration of 0.5µg/ml. The portion of gel containing the fragment of the expected length was withdrawn. In order to extract the DNA, 10µl of Qiaex resin (Qiaex extraction kit, firm Qiagen) were added for each 200mg of gel. The supplier's method was then followed. The DNA was then supplemented with a tenth of a volume of 10XH buffer (Boehringer) and 20 units of Eco RI enzyme (Boehringer). After incubation overnight at 37°C, the DNA was precipitated with 0.1 volumes of 5M NH_4OAc and one volume of isopropanol. After 10 minutes at ambient temperature, the DNA was centrifuged for 20 minutes at 14000 rpm and the precipitate was washed with 70% ethanol. The DNA was resuspended in 15µl of H_2O. The concentration was determined on gel by comparison with a known standard.

The amplified fragment was inserted in the pUC18 vector. A ligation mixture was prepared as follows: 1µl pUC18 plasmid DNA cut with Eco RI (20ng), 1.5µl fragment amplified with NOCC1 and 4 (25ng), 1µl 10X ligase buffer (Boehringer), 1µl T4 DNA ligase (1U/µl) (Boehringer), 4.5µl H_2O. The reaction mixture was incubated at 14°C for 12 hours.

In order to prepare competent cells, the method based on the compound hexamino-cobalt chloride was used (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.76-1.81). 10µl of the ligation mixture were added to each aliquot of competent cells, defrosted on ice. After the cells had been incubated on ice for 30 minutes they were subjected to thermal shock at 42°C for 90 seconds and were then replaced in ice for 60 seconds. After the addition of 0.5 ml of SOC broth (2% Bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM glucose, pH7), the cells were incubated at 37°C with stirring for 90'. 100, 200 and 300 µl aliquots were spread on plates containing solid LB broth (10gr/l NaCl, 10gr/l Bactotryptone, 5gr/l yeast extract, pH7.5, 15gr/l agar) with the addition of 50µg/ml of ampicillin and in the presence of IPTG and X-Gal. The plates were then incubated at 37°C overnight.

Some of the bacterial colonies obtained were first analyzed for their plasmid content by a quick method (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.32). The colonies containing a plasmid of the expected length were grown and their plasmid DNA extracted (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.33). Those containing a fragment of the expected length (1160 bp) were identified by digestion of the plasmid DNA with Eco RI. The E1 colony was selected.

One end of the insert of the E1 colony was sequenced. The plasmid DNA of the E1 clone was denatured and partially sequenced by Sanger's method using the enzyme Sequenase and ^{35}S-dATP (Amersham). The sequencing products were separated on 8% acrylamide, 8M urea, 1XTBE gel. After electrophoresis, the gel was dried and exposed overnight in contact with an autoradiographic plate (β max, Amersham). The sequence was compared with that published and was identical, identifying the *Arabidopsis* FAD2 gene in the cloned fragment.

Extraction of nucleic acids from hazel

Hazelnuts of the Nocchione, Montebello and San Giovanni varieties were harvested when almost fully ripe. The kernel was skinned before being used or frozen in liquid nitrogen. The leaves were harvested at a young stage and frozen in liquid nitrogen. 3 ml of extraction buffer were used for each gram of vegetable material with the use of the method described by Verwoerd et al. (Nucl. Ac. Res., 1989, 2362). Upon completion of the extraction, two selective precipitations were carried out by the addition of NaCl 2M, and 2 volumes of 95% ethanol to eliminate polysaccharides. The final pellet was resuspended in H_2O. Further centrifuging was then carried out to eliminate any non-resuspended material.

On the other hand, DNA was extracted from young leaves of the Nocchione and Montebello varieties. The vegetable tissue was pulverized in liquid nitrogen and the DNA extracted by the CTAB (REF) method. To eliminate the polysaccharides, NaCl 2M and 2 volumes of 95% ethanol were added. The samples were incubated for 15' at -80°C and centrifuged for 15' at 4°C and 14000 rmp (Eppendorf). This selective precipitation was repeated twice and the final pellet was resuspended in H_2O. Further centrifuging was then carried out to eliminate any non-resuspended material.

Checking of the probe on hazel DNA and RNA

About 20µg of DNA of the Montebello and Nocchione varieties was cut with Eco RI restriction enzyme in a volume of 300 µl in the presence of 400 units of enzyme and H buffer (Promega), with incubation for one night at 37°C. After digestion had been checked by gel electrophoresis of one twentieth of the reaction mixture, the samples were precipitated with ethanol and resuspended in 30µl of H_2O. The DNA was then subjected to electrophoresis on 0.7% agarose gel and transferred by capillarity onto nylon membrane (Southern blot) for one night in the presence of 20 x SSC (3M NaCl, 0.3M Na citrate). The membrane was dried in air for 30' and then fixed by UV treatment (120,000 µJ/cm^2).

The *Arabidopsis* Δ12 desaturase gene was used as a probe. For this purpose, the plasmid DNA of the E1 clone (5µg) was cut with 20 units of Eco RI in the presence of H buffer (Boehringer) in a volume of 30µl for 12 hours at 37°C. The insert of the clone was separated from the vector by electrophoresis on 1% agarose gel and extracted from the gel with the use of Qiaex resin in accordance with the suppliers' instructions (Qiagen). The DNA was denatured for 10' at 100°C, cooled rapidly in dry ice, and marked by the random priming method with the use of 6000 Ci/mmol (α^{32})P dATP and the reagents of Boehringer's marking kit.

The nylon membrane containing the hazel DNA was prehybridized for 1.5 hours at 55°C in standard buffer (5 x SSC, 0.1% (w/v) N-laurylsarcosine, 0.02% SDS, 1% blocking reagent solution) (10% blocking reagent solution: 10gr Boehringer blocking reagent in 150mM NaCl, 100mM maleic acid, pH7.5). The membrane was then hybridized with the *Arabidopsis* probe for one night at 55°C. The non-hybridized probe was washed twice for 15' in 2 x SSC, 0.1% SDS and twice for 15' each in 0.3 x SSC, 0.1% SDS, always at a temperature of 55°C. The probe remained coupled to the homologous sequences on the membrane was detected by autoradiography.

The RNA extracted from the young leaves of the Montebello and Nocchione varieties and from the kernels of the San Giovanni variety was separated on denaturing gel in the presence of formamide and transferred to nylon membrane by Northern blotting (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 7.43-7.45). 40µg/sample of total RNA extracted from San Giovanni kernels, Nocchione leaves and Montebello leaves were used. 60 pg of probe were used as a positive control. The RNA was loaded onto a 1% agarose gel in the presence of formaldehyde. The samples were then subjected to electrophoresis for 3 hours at 80 volts in the presence of 1xMOPS. The gel was rinsed in H_2O and then stained with ethidium bromide 0.5 µg/ml to display the RNA. The RNA was then transferred onto a nylon membrane (Boehringer) by "capillary blotting" in the presence of 20 x SSC throughout the night at 4°C. After transfer, the membrane was dried on 3 MM paper and then fixed by crosslinking using UV light (Stratagene UV Stratalinker 120000 µJ/cm^2). The RNA was hybridised with the *Arabidopsis* Δ12 desaturase probe as described for the DNA. Detection was carried out by autoradiography. The heterologous *Arabidopsis* probe was able to display a band with a molecular weight of about 1500 bp in the hazel RNA and 3 bands of about 18, 8 and 2.8 kb in the hazel DNA cut with Eco RI.

Construction of a gene library of cDNA

The gene library of cDNA was constructed from RNA from kernels harvested when almost fully ripe and taken from plants of the San Giovanni variety. For this purpose, the Poly(A)+mRNA was isolated from the total RNA with the use of the Poly(A)Tract mRNA Isolation System II, in accordance with the method provided by the firm Promega. The samples were eluted in H_2O and precipitated with 0.1 volumes of 3M NaOAc and 3 volumes of 95% ethanol. After one night at -80°C, the RNA was centrifuged for 15' at 14000 rpm (Eppendorf), the pellet was rinsed in 75% ethanol and resuspended in 10µl of H_2O. The concentration was read with a spectrophotometer and the yield was 3.2µg of Poly(A)+mRNA per mg of total RNA.

The messenger RNA polyadenilate derived from kernels of the San Giovanni variety was used as a template for the synthesis of complementary DNA (cDNA) with the use of Boehringer's "cDNA synthesis kit" in accordance with the method recommended by the suppliers. An extraction was then carried out with one volume of phenol:chloroform: isoamyl alcohol (25:24:1). The cDNA was then purified in a Pharmacia column (cDNA spun columns) after the addition of NaCl 100 mM. The buffer used was the following: 10mM Tris-HCl pH 7.5, 1mM EDTA, 150mM NaCl. Eco RI "adaptors" (Pharmacia) were added to the ends of the cDNA. The reaction mixture contained: 5µl of cDNA (half of the cDNA obtained from 6µg of Poly(A)+RNA), 10µl of ligase buffer 10 x (Promega), 10µl of Eco RI adaptors (0.01u/µl), 6 units of T4 DNA ligase (Promega), in a final volume of 100µl. After incubation for 12 hours at 12°C, the ligase enzyme was inactivated for 10' at 65°C. Phosphorylation of the adaptors then followed by the addition, to the 100µl mixture, of 10µl of 100mM ATP and 10 units of T4 polynucleotide kinase. After incubation at 37°C for 30', the enzyme was inactivated by incubation for 10' at 65°C. Purification was then carried out with one volume of phenol:chloroform:isoamyl alcohol (25:24:1). The cDNA was then purified from fragments of less than 400 bp as follows. After the addition of NaCl to a final concentration of 0.1M NaCl, the cDNA was separated by chromatography in a column with Sepharose CL-4B resin (Size prep 400 spun column, Pharmacia) according to the method suggested by the suppliers. The fragments of cDNA shorter than 400 bases were thus excluded. The cDNA was precipitated with one thirtieth of a volume of 3M NaOAc and 2 volumes of 95% ethanol, centrifuged and resuspended in 10µl of H_2O.

The cDNA was inserted in the λ phage vector Zap II cut with Eco RI and dephosphorylated (Stratagene) in the following manner: 2μl of cDNA (200 ng), 1μl of λ Zap II cut with Eco RI (1μg/μl) (Stratagene), 0.5μl of T4 DNA Ligase (4U/μl) (Promega), 0.5μl of 10 x ligation buffer (Promega), 1μl of H_2O. The reaction mixture was incubated for 14 hours at 12°C. The mixture containing the cDNA inserted in the vector was used for the reconstruction of the phages with the use of Stratagene's Gigapack Gold "in vitro packaging" kit. The gene library of phages thus obtained was constituted by about 300,000 pfu (plaque-forming units). In order to amplify the gene library, XL1 Blue MRF' cells were prepared as described by Stratagene and used the same day. The gene libraries were plated at a concentration of about 5000 pfu per plate (95 cm^2). After growth, the phages were resuspended in SM (5.8gr/l NaCl, 2gr/l $MgSO_4.7H_2O$, 50ml/l 1M Tris HCl (pH 7.5), 5ml/l 2% gelatine) and, after the addition of chloroform to 5% and incubation for 15 minutes at ambient temperature, the cell debris was centrifuged for 10 minutes at 2000 x g. Chloroform to 0.3% was added to the supernatant liquid and the phages were preserved at 4°C. Aliquots were preserved at -70°C after the addition of DMSO to 7%. The gene library was titled.

Construction of a partial genome gene library

The DNA of the Nocchione variety was digested with Eco RI restriction enzyme and separated on agarose gel. The fragments with lengths of up to 10000 bp (base pairs) were isolated from the gel with the use of Qiaex resin according to the Qiagen's method. For cloning in the λ vector Zap II, 400ng of DNA fragments were incubated with 1μg of desphosphorylated λ Zap II (Stratagene) in the presence of ligase buffer and 1.5 units of T4 DNA ligase (Promega) for 12 hours at 14°C.

Strategene's Gigapack Gold "in vitro packaging" kit was used in accordance with the suppliers' instructions to make up the gene library. The gene library of phages thus produced was amplified as described for the cDNA gene library. The complexity of the gene library was 1,500,000 clones. This gene library was also amplified.

Screening of the cDNA gene library

About 250,000 phages of the cDNA gene library were plated on LB broth in the presence of XL1 Blue MRF' cells, divided into 12 plates each containing 20,000 pfu. After growth, the phages were transferred onto nylon membranes and their denatured DNA was fixed on the membranes as described by Boehringer for screening with non-radioactive probes. The membranes were then hybridized with the *Arabidopsis* Δ12 desaturase gene. The probe was prepared by the isolation of the insert containing the entire coding region of the gene from the plasmid. The insert was then marked with digoxigenin-dUTP with the use of Boehringer's "DNA labelling kit". Prehybridization was carried out in standard buffer (Boehringer) and hybridization was carried out in the same buffer with the addition of the *Arabidopsis* probe at a concentration of 10ng/ml and at a temperature of 55°C.

After washing twice in 2xSSC, 0.1% SDS for 5 minutes at ambient temperature and washing twice in 0.3xSSC, 0.1%SDS at 55°C, detection was carried out with the use of an anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer) and a chemiluminescent substrate (AMPPD, Boehringer).

11 positive phage plaques were identified. These were isolated, the phages resuspended in SM and titled. From 50 to 200 phages were plated for each positive plaque. The plaques were transferred onto nylon membranes and subjected to a second hybridization with the *Arabidopsis* Δ12 desaturase probe, as already described above. The following clones which could hybridize with the *Acabidopsis* Δ12 desaturase gene were obtained from the second screening: I, F, 4.

Screening of the genome gene library

The gene library of Nocchione DNA was subjected to screening in the same way as the cDNA gene library. 1,600,000 phages were plated, divided into 40 plates. After growth, they were transferred to nylon membranes as described for the cDNA gene library. The membranes were then hybridized with the *Arabidopsis* Δ12 desaturase gene as described for the cDNA gene library. Autoradiography of the membranes showed 9 positive plaques. These plaques were isolated, titled and subjected to a second screening. 6 plaques were re-confirmed as positive. 4 of these gave a very strong signal.

Analysis of the clones isolated

The following positive phage clones were converted into plasmids by *in vivo* excision in accordance with the method suggested by Stratagene (Gigapack Gold in vitro packaging) : I, F, 4 (cDNA gene library), N2, N11, N17, N18, N21, N25 (genome gene library).

The plasmid DNA of the clones of the cDNA gene library was isolated and the length of the insert analyzed by digestion with Eco RI. The plasmid DNA of the genome clones was isolated, the length of the insert analyzed by cutting

with restriction enzyme, and the clones rechecked by hybridization with the *Arabidopsis* probe. Figure 1 shows the map of the N2 genome clone.

Sequencing

The N2 clone was selected from the genome clones. For sequencing, the insert was fragmented with Sau3A restriction enzyme and the fragments obtained were subcloned in pUC18 vector cut with BamHI (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.68-1.69). The clones obtained were analyzed both for the length of the insert and by hybridization with the *Arabidopsis* probe. Since the N2 insert was 2.8 kb and hence longer than the Δ12 desaturase gene, the hybridization excluded the clones containing sequences outside the gene. The insert of the I, F, 4 and N2 clones was isolated and sequenced with the use of the Sequenase kit and (35S)dATP. All of the clones (cDNA and genome) were first sequenced at the ends with the use of primers which could couple with the vector in both orientations. In order to complete the internal regions and to assemble the fragments of the N2 genome clone, internal oligonucleotides were then designed and synthesized and were used for the sequencing. The following table shows the sequences of the internal oligonucleotides:

OLIGONUCLEOTIDE	SEQUENCE
N2-3SS	CAG ACC AGC ATC CGA GAC
N2-3SD	GGA TTG GCT TAG GGG GGC
N2-29R'S	GCC AAC CAT GTC ATC AAC CC
NOCCS	ATG GTA GAG AAG AGA TGG TG
COL	CTG GTG GGT TGT TGA AG
N2-S1N	GGA GAG GTC ATA AAC AAC

The I and F clones were sequenced entirely. As far as the N2 clone is concerned, only the regions corresponding to the gene were sequenced. Figures 2 and 3 show their sequence. The I and F cDNA clones were identical. A comparison between I and the N2 genome clone showed the same sequence (Fig. 4), indicating that N2 contains the gene which codes for the cDNA of the I clone.

Comparison between the gene isolated and other desaturases

The nucleotide and amino-acid sequence of the N2 clone was compared with other desaturases (Figure 6). The greatest homology was with the two Δ12 desaturases of the endoplasmic reticulum and with a hydroxylase of ricin which uses the same substrate as Δ12 desaturase. Homology with the plastid Δ12 desaturases and with both the plastid and endoplasmic reticulum Δ15 desaturases was, however, much lower. Figure 5 shows the comparison between the amino-acid sequence of hazel Δ12 and those of *Arabidopsis* and soya.

Checking of the expression of the hazel Δ12 desaturase gene

RNA was extracted from kernels of the San Giovanni, Montebello and Nocchione varieties and from leaves of the Montebello and Nocchione varieties. After separation on agarose gel, the RNA was transferred onto a nylon membrane and hyhridized with the insert of the I clone marked with digoxigenin. The result is shown in Figure 7, in which a band is visible in the kernel RNA but not in that of the leaves.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: SOREMARTEC S.A.
(B) STREET: Dreve de l'Arc-en-Ciel 102
(C) CITY: Arlon-Schoppach
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): 6700

(ii) TITLE OF INVENTION: Isolation and sequencing of the hazel FAD2-N gene

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: CH 0550/96
(B) FILING DATE: 04-MAR-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1662 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Corylus avellana cv. Nocchione
(F) TISSUE TYPE: leaves

(vii) IMMEDIATE SOURCE:
(B) CLONE: N2

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:222..1370
(D) OTHER INFORMATION:/product= "delta-12 desaturase"
/gene= ""Fad2""

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CCTCATAAAA AAGTAAGCTC ATTTACCTCA AGTAGGGTTT CCTTATGACA AATGAGTCCC
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    180

AAGTTGATTT TCTCCAGCAT TGGACATAGC CTCTGTAGAC A ATG GGA GCT AGA
      233
                                              Met Gly Ala Arg
                                                1

AGC CGA ATG CCT GCT ACC AAC AAG CCT AAA GAG CAA AAA ACA CCC ATC
     281
Ser Arg Met Pro Ala Thr Asn Lys Pro Lys Glu Gln Lys Thr Pro Ile
 5                  10                  15                  20

CAG CGA GCA CCA CAC ACA AAA CCC CCA TTC ACT CTT AGC CAA CTC AAG
     329
Gln Arg Ala Pro His Thr Lys Pro Pro Phe Thr Leu Ser Gln Leu Lys
                25                  30                  35

AAA GCC GTC CCA CCC AAT TGT TTC CAA CGC TCT CTC CTA CGC TCG TTC
     377
Lys Ala Val Pro Pro Asn Cys Phe Gln Arg Ser Leu Leu Arg Ser Phe
            40                  45                  50

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        55                  60                  65

GCT ACC TCT TAC TTC CAT CTC CTC CCT CAC CCC CTT TCC TAC TTG GCA
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Ala Thr Ser Tyr Phe His Leu Leu Pro His Pro Leu Ser Tyr Leu Ala
     70                  75                  80

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     521
Trp Ser Ile Tyr Trp Ala Leu Gln Gly Cys Ile Leu Thr Gly Val Trp
 85                  90                  95                 100
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569
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105 110 115

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120 125 130

TAC TTT TCA TGG AAG ATT AGC CAC TGT CGC CAC CAC TCT AAC ACC GGC
665
Tyr Phe Ser Trp Lys Ile Ser His Cys Arg His His Ser Asn Thr Gly
135 140 145

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713
Ser Leu Asp Arg Asp Glu Val Phe Val Pro Lys Pro Lys Ser Lys Met
150 155 160

CCA TGG TTT TCT AAG TAC TTC AAC AAC CCA CCA GGT AGG GTC CTC ACT
761
Pro Trp Phe Ser Lys Tyr Phe Asn Asn Pro Pro Gly Arg Val Leu Thr
165 170 175 180

CTT TTG ATC ACA CTC ACT CTA GGC TGG CCC TTG TAC TTA GCC TTG AAT
809
Leu Leu Ile Thr Leu Thr Leu Gly Trp Pro Leu Tyr Leu Ala Leu Asn
185 190 195

GTT TCT GGC CGA CCC TAT GAT CGT TTT GCT TGC CAC TAT GAT CCC TAT
857
Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys His Tyr Asp Pro Tyr
200 205 210

GGC CCC ATT TAT TCC AAT CGC GAA AGG TGT CAA ATA TTT GTC TCG GAT
905
Gly Pro Ile Tyr Ser Asn Arg Glu Arg Cys Gln Ile Phe Val Ser Asp
215 220 225

GCT GGT GTC TTT GCT ACA ACT TAT GTG CTT TAC TAC GCA GCA ATG TCA
953

Ala Gly Val Phe Ala Thr Thr Tyr Val Leu Tyr Tyr Ala Ala Met Ser
230 235 240

AAA GGG CTG GCA TGG CTT GTA TTC ATT TAT GGT ATG CCA TTG CTC ATA
1001
Lys Gly Leu Ala Trp Leu Val Phe Ile Tyr Gly Met Pro Leu Leu Ile
245 250 255 260

GTG AAT GGC TTC CTT GTA TTA ATC ACC TAC TTG CAG CAC ACT CAC CCT
1049
Val Asn Gly Phe Leu Val Leu Ile Thr Tyr Leu Gln His Thr His Pro
265 270 275

GCA TTG CCG CAC TAT GAC TCA TCA GAA TGG GAT TGG CTT AGG GGG GCA
1097
Ala Leu Pro His Tyr Asp Ser Ser Glu Trp Asp Trp Leu Arg Gly Ala
280 285 290

TTG GCG ACG GCG GAT AGA GAT TAC GGA ATG CTG AAT AAG GTT TTC CAC
1145
Leu Ala Thr Ala Asp Arg Asp Tyr Gly Met Leu Asn Lys Val Phe His
295 300 305

AAT ATC ATA GAC ACC CAT GTG GCT CAC CAT CTC TTC TCT ACC ATG CCT
1193
Asn Ile Ile Asp Thr His Val Ala His His Leu Phe Ser Thr Met Pro
310 315 320

CAT TAC CAT GCA ATG GAA GCC ACC AAA GCA ATC AAG TCA ATA TTG GGC
1241
His Tyr His Ala Met Glu Ala Thr Lys Ala Ile Lys Ser Ile Leu Gly
325 330 335 340

AAA TAC TAC CAG TTT GAT GGC ACT CCA GTT TAC AAG GCA GTG TGG AGG
1289
Lys Tyr Tyr Gln Phe Asp Gly Thr Pro Val Tyr Lys Ala Val Trp Arg
345 350 355

GAG GCT AAA GAG TGC CTT TAT GTT GAG TCG GAC GAG GGG GCC CCT AAC
1337
Glu Ala Lys Glu Cys Leu Tyr Val Glu Ser Asp Glu Gly Ala Pro Asn

```
                    360                 365                 370

AAA GGT GTT TTC TGG TAT CAG AGC AAG CTG TGA TATTGGCTGG ATAGAGCCAA
  1390
Lys Gly Val Phe Trp Tyr Gln Ser Lys Leu  *

        375                 380

AGAAAATGTG ATTAGTAAGG TAGTGTCTTT GGTCAGTTTG GTGTGTTAAG GAACAAATAA
  1450

TAATAATTAG CGACTATGAA TAGTTATTGT TAAACAAAAT TCACCCTTAT GTTTAGCAGG
  1510

AACTTTTCTG GCTACACTTT TTTTCGTATG AAAAGCGCAT ATTTTTTAAT TGTTATATTG
  1570

TTTTGACATT ACTCAAGCTT CAAAATTAAT ATCACAGAAA ATATCCAATG TCGAAGGTTT
  1630

CATTGTAGGT TGAAAACTTT ATATTGAGGT GG
     1662


(2) INFORMATION FOR SEQ ID NO: 2:

       (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 383 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Gly Ala Arg Ser Arg Met Pro Ala Thr Asn Lys Pro Lys Glu Gln
  1               5                  10                  15

Lys Thr Pro Ile Gln Arg Ala Pro His Thr Lys Pro Pro Phe Thr Leu
             20                  25                  30

Ser Gln Leu Lys Lys Ala Val Pro Pro Asn Cys Phe Gln Arg Ser Leu
         35                  40                  45

Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ser Leu Ala Phe Leu
     50                  55                  60

Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Leu Leu Pro His Pro Leu
 65                  70                  75                  80

Ser Tyr Leu Ala Trp Ser Ile Tyr Trp Ala Leu Gln Gly Cys Ile Leu
                 85                  90                  95
```

```
Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe Ser
            100                 105                 110

Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu Thr Leu His Ser Ala
        115                 120                 125

Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser His Cys Arg His His
    130                 135                 140

Ser Asn Thr Gly Ser Leu Asp Arg Asp Glu Val Phe Val Pro Lys Pro
145                 150                 155                 160

Lys Ser Lys Met Pro Trp Phe Ser Lys Tyr Phe Asn Asn Pro Pro Gly
                165                 170                 175

Arg Val Leu Thr Leu Leu Ile Thr Leu Thr Leu Gly Trp Pro Leu Tyr
            180                 185                 190

Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys His
        195                 200                 205

Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg Glu Arg Cys Gln Ile
    210                 215                 220

Phe Val Ser Asp Ala Gly Val Phe Ala Thr Thr Tyr Val Leu Tyr Tyr
225                 230                 235                 240

Ala Ala Met Ser Lys Gly Leu Ala Trp Leu Val Phe Ile Tyr Gly Met
                245                 250                 255

Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile Thr Tyr Leu Gln
            260                 265                 270

His Thr His Pro Ala Leu Pro His Tyr Asp Ser Ser Glu Trp Asp Trp
        275                 280                 285

Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp Tyr Gly Met Leu Asn
    290                 295                 300

Lys Val Phe His Asn Ile Ile Asp Thr His Val Ala His His Leu Phe
305                 310                 315                 320

Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala Ile Lys
                325                 330                 335

Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp Gly Thr Pro Val Tyr Lys
```

```
                340                      345                      350

Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val Glu Ser Asp Glu
         355                     360                     365

Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln Ser Lys Leu  *
    370                     375                     380
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1133 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Corylus avellana L. cv. San Giovanni
(D) DEVELOPMENTAL STAGE: Seed, storage deposition stage

(vii) IMMEDIATE SOURCE:
(B) CLONE: I

(ix) FEATURE:
(A) NAME/KEY: mRNA
(B) LOCATION:1..1133
(D) OTHER INFORMATION:/partial
/gene= "Fad2"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1019
(D) OTHER INFORMATION:/partial
/codon_start= 3
/product= "delta-12 desaturase"
/gene= "Fad2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
TC CAA CGC TCT CTC CTA CGC TCG TTC TCA TAT GTT GTT TAT GAC CTC
         47
   Gln Arg Ser Leu Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu

         385                     390                     395

TCC TTA GCC TTC CTC TTC TAC TAT ATT GCT ACC TCT TAC TTC CAT CTC
```

```
 95
Ser Leu Ala Phe Leu Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Leu

    400                 405                 410

CTC CCT CAC CCC CTT TCC TAC TTG GCA TGG TCA ATC TAT TGG GCT CTC
    143
Leu Pro His Pro Leu Ser Tyr Leu Ala Trp Ser Ile Tyr Trp Ala Leu

415                 420                 425                 430

CAA GGC TGC ATT CTC ACC GGC GTT TGG GTC ATC GCA CAT GAG TGC GGT
    191
Gln Gly Cys Ile Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly

                435                 440                 445

CAC CAT GCC TTT AGT GAC TAC CAA TGG GTT GAT GAC ATG GTT GGC CTA
    239
His His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu

            450                 455                 460

ACC CTT CAC TCT GCT CTT TTA GTT CCA TAC TTT TCA TGG AAG ATT AGC
    287
Thr Leu His Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser

        465                 470                 475

CAC TGT CGC CAC CAC TCT AAC ACC GGC TCC CTT GAC CGA GAT GAG GTG
    335
His Cys Arg His His Ser Asn Thr Gly Ser Leu Asp Arg Asp Glu Val

    480                 485                 490

TTT GTC CCC AAG CCG AAA TCC AAA ATG CCA TGG TTT TCT AAG TAC TTC
    383
Phe Val Pro Lys Pro Lys Ser Lys Met Pro Trp Phe Ser Lys Tyr Phe

495                 500                 505                 510

AAC AAC CCA CCA GGT AGG GTC CTC ACT CTT TTG ATC ACA CTC ACT CTA
    431
Asn Asn Pro Pro Gly Arg Val Leu Thr Leu Leu Ile Thr Leu Thr Leu

                515                 520                 525

GGC TGG CCC TTG TAC TTA GCC TTG AAT GTT TCT GGC CGA CCC TAT GAT
    479
Gly Trp Pro Leu Tyr Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp
```

530 535 540

CGT TTT GCT TGC CAC TAT GAT CCC TAT GGC CCC ATT TAT TCC AAT CGC
527
Arg Phe Ala Cys His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg

545 550 555

GAA AGG TGT CAA ATA TTT GTC TCG GAT GCT GGT GTC TTT GCT ACA ACT
575
Glu Arg Cys Gln Ile Phe Val Ser Asp Ala Gly Val Phe Ala Thr Thr

560 565 570

TAT GTG CTT TAC TAC GCA GCA ATG TCA AAA GGG CTG GCA TGG CTT GTA
623
Tyr Val Leu Tyr Tyr Ala Ala Met Ser Lys Gly Leu Ala Trp Leu Val

575 580 585 590

TTC ATT TAT GGT ATG CCA TTG CTC ATA GTG AAT GGC TTC CTT GTA TTA
671
Phe Ile Tyr Gly Met Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu

595 600 605

ATC ACC TAC TTG CAG CAC ACT CAC CCT GCA TTG CCG CAC TAT GAC TCA
719
Ile Thr Tyr Leu Gln His Thr His Pro Ala Leu Pro His Tyr Asp Ser

610 615 620

TCA GAA TGG GAT TGG CTT AGG GGG GCA TTG GCG ACG GCG GAT AGA GAT
767
Ser Glu Trp Asp Trp Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp

625 630 635

TAC GGA ATG CTG AAT AAG GTT TTC CAC AAT ATC ATA GAC ACC CAT GTG
815
Tyr Gly Met Leu Asn Lys Val Phe His Asn Ile Ile Asp Thr His Val

640 645 650

GCT CAC CAT CTC TTC TCT ACC ATG CCT CAT TAC CAT GCA ATG GAA GCC
863
Ala His His Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala

655 660 665 670

```
ACC AAA GCA ATC AAG TCA ATA TTG GGC AAA TAC TAC CAG TTT GAT GGC
    911
Thr Lys Ala Ile Lys Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp Gly

                675                 680                 685

ACT CCA GTT TAC AAG GCA GTG TGG AGG GAG GCT AAA GAG TGC CTT TAT
    959
Thr Pro Val Tyr Lys Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr

            690                 695                 700

GTT GAG TCG GAC GAG GGG GCC CCT AAC AAA GGT GTT TTC TGG TAT CAG
    1007
Val Glu Ser Asp Glu Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln

        705                 710                 715

AGC AAG CTG TGA TATTGGCTGG ATAGAGCCAA AGAAAATGTG ATTAGTAAGG
     1059
Ser Lys Leu  *
    720

TAGTGTCTTT GGTCAGTTTG GTGTGTTAAG GAACAAATAA TAATAATTAG CGACTATGAA
  1119

TAGTTATTGT TAAA
     1133
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 339 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Gln Arg Ser Leu Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ser
  1               5                  10                  15

Leu Ala Phe Leu Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Leu Leu
             20                  25                  30

Pro His Pro Leu Ser Tyr Leu Ala Trp Ser Ile Tyr Trp Ala Leu Gln
         35                  40                  45

Gly Cys Ile Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His
```

50 55 60

His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu Thr
65 70 75 80

Leu His Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser His
85 90 95

Cys Arg His His Ser Asn Thr Gly Ser Leu Asp Arg Asp Glu Val Phe
100 105 110

Val Pro Lys Pro Lys Ser Lys Met Pro Trp Phe Ser Lys Tyr Phe Asn
115 120 125

Asn Pro Pro Gly Arg Val Leu Thr Leu Leu Ile Thr Leu Thr Leu Gly
130 135 140

Trp Pro Leu Tyr Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg
145 150 155 160

Phe Ala Cys His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg Glu
165 170 175

Arg Cys Gln Ile Phe Val Ser Asp Ala Gly Val Phe Ala Thr Thr Tyr
180 185 190

Val Leu Tyr Tyr Ala Ala Met Ser Lys Gly Leu Ala Trp Leu Val Phe
195 200 205

Ile Tyr Gly Met Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile
210 215 220

Thr Tyr Leu Gln His Thr His Pro Ala Leu Pro His Tyr Asp Ser Ser
225 230 235 240

Glu Trp Asp Trp Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp Tyr
245 250 255

Gly Met Leu Asn Lys Val Phe His Asn Ile Ile Asp Thr His Val Ala
260 265 270

His His Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr
275 280 285

Lys Ala Ile Lys Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp Gly Thr
290 295 300

```
Pro Val Tyr Lys Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val
305                 310                 315                 320

Glu Ser Asp Glu Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln Ser
                325                 330                 335

Lys Leu  *
```

Claims

1. A fragment of DNA from hazel (*Corylus avellana L.*) comprising the nucleotide sequence shown in Figure 2.

2. A DNA fragment comprising the nucleotide sequence shown in Figure 2 from base 222 to base 1367, which codes for the hazel Δ12 desaturase enzyme of the endoplasmic reticulum or for a homologous sequence which can code for the same amino-acid sequence.

3. A nucleotide sequence coding for a protein or peptide having an amino-acid homology greater than or equal to 80% and preferably greater than 90% with the hazel Δ12 desaturase enzyme of the endoplasmic reticulum of Claim 2 and having the function of the said enzyme.

4. A recombinant DNA sequence comprising a DNA sequence according to Claims 1, 2 and 3, or a portion of such a sequence, together with sequences regulating expression.

5. A recombinant DNA molecule comprising a cloning vector in which a DNA sequence according to any one of Claims 1, 2, 3 and 4 is inserted.

6. A DNA molecule according to Claim 5, in which the cloning vector is a plasmid or a phage.

7. A DNA molecule according to Claim 4 or Claim 5 having the restriction map shown in Figure 1.

8. A host organism including a recombinant DNA molecule according to any one of Claims 3 to 6.

9. A host organism according to Claim 8, selected from a vegetable cell,an animal cell, and a micro-organism.

10. A genetically modified organism capable of expressing the FAD2-N gene, having the amino-acid sequence shown in Figure 2 from bp 222 to bp 1367, portions of this gene, or this gene conjugated with other molecules and containing sequences which can inactivate endogenous genes.

11. A hazel Δ12 desaturase enzyme of the endoplasmic reticulum having the amino-acid sequence shown in Figure 2 in substantially pure form.

12. A fusion polypeptide comprising the amino-acid sequence of the enzyme of Claim 11, in which the amino-acids additively connected thereto do not interfere with the desaturase activity or can easily be eliminated.

13. The use of the FAD2-N gene coding for the hazel Δ12 desaturase enzyme of the endoplasmic reticulum or of portions thereof for the isolation of enzymes having the function of hazel desaturase or of the desaturase of another species.

14. The use of the nucleotide sequences of the FAD2-N gene shown in Figure 2 for the construction of expression systems which can alter the fatty-acid content in hazel.

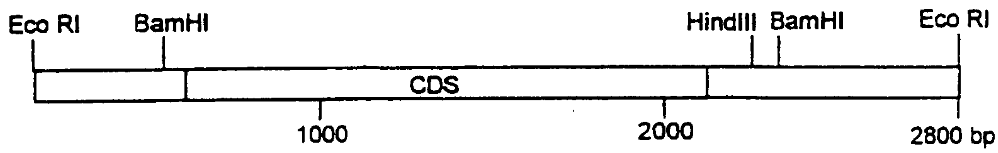

Fig. 1 - Restriction map of the genomic clone "N2". CDS: coding region; bp: base pair.

Fig. 2 - Nucleotide sequence of the gene FAD2-N corresponding to an internal fragment of the genomic clone "N2". Aminoacid residues of the coding region are also reported.

CCTCATAAAAAAGTAAGCTCATTTACCTCAAGTAGGGTTTCCTTATGACAAATGAGTCCC 60
GGAGTATTTTTTCATTCGAGTAAATGGAGTTCATCCCAAAGGAATACTGTTTACTCAGGG

GCAATCCTTTTCTATGAGGTGCTATAATTGCAAATGTCCAAATCATAGGGATATGGATCC 120
CGTTAGGAAAAGATACTCCACGATATTAACGTTTACAGGTTTAGTATCCCTATACCTAGG

AAATACTATTAATATTATGTAGTGTGTTTTTTTTTTTCCCTCAAATTTACTCTCACACCT 180
TTTATGATAATTATAATACATCACACAAAAAAAAAAAGGGAGTTTAAATGAGAGTGTGGA

AAGTTGATTTTCTCCAGCATTGGACATAGCCTCTGTAGACAATGGGAGCTAGAAGCCGAA 240
TTCAACTAAAAGAGGTCGTAACCTGTATCGGAGACATCTGTTACCCTCGATCTTCGGCTT
Met Gly Ala Arg Ser Arg

TGCCTGCTACCAACAAGCCTAAAGAGCAAAAAACACCCATCCAGCGAGCACCACACACAA 300
ACGGACGATGGTTGTTCGGATTTCTCGTTTTTTGTGGGTAGGTCGCTCGTGGTGTGTGTT
Met Pro Ala Thr Asn Lys Pro Lys Glu Gln Lys Thr Pro Ile Gln Arg Ala Pro His Thr

AACCCCCATTCACTCTTAGCCAACTCAAGAAAGCCGTCCCACCCAATTGTTTCCAACGCT 360
TTGGGGGTAAGTGAGAATCGGTTGAGTTCTTTCGGCAGGGTGGGTTAACAAAGGTTGCGA
Lys Pro Pro Phe Thr Leu Ser Gln Leu Lys Lys Ala Val Pro Pro Asn Cys Phe Gln Arg

CTCTCCTACGCTCGTTCTCATATGTTGTTTATGACCTCTCCTTAGCCTTCCTCTTCTACT 420
GAGAGGATGCGAGCAAGAGTATACAACAAATACTGGAGAGGAATCGGAAGGAGAAGATGA
Ser Leu Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ser Leu Ala Phe Leu Phe Tyr

ATATTGCTACCTCTTACTTCCATCTCCTCCCTCACCCCCTTTCCTACTTGGCATGGTCAA 480
TATAACGATGGAGAATGAAGGTAGAGGAGGGAGTGGGGGAAAGGATGAACCGTACCAGTT
Tyr Ile Ala Thr Ser Tyr Phe His Leu Leu Pro His Pro Leu Ser Tyr Leu Ala Trp Ser

TCTATTGGGCTCTCCAAGGCTGCATTCTCACCGGCGTTTGGGTCATCGCACATGAGTGCG 540
AGATAACCCGAGAGGTTCCGACGTAAGAGTGGCCGCAAACCCAGTAGCGTGTACTCACGC
Ile Tyr Trp Ala Leu Gln Gly Cys Ile Leu Thr Gly Val Trp Val Ile Ala His Glu Cys

GTCACCATGCCTTTAGTGACTACCAATGGGTTGATGACATGGTTGGCCTAACCCTTCACT 600
CAGTGGTACGGAAATCACTGATGGTTACCCAACTACTGTACCAACCGGATTGGGAAGTGA
Gly His His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu Thr Leu His

CTGCTCTTTTAGTTCCATACTTTTCATGGAAGATTAGCCACTGTCGCCACCACTCTAACA 660
GACGAGAAAATCAAGGTATGAAAAGTACCTTCTAATCGGTGACAGCGGTGGTGAGATTGT
Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser His Cys Arg His His Ser Asn

CCGGCTCCCTTGACCGAGATGAGGTGTTTGTCCCCAAGCCGAAATCCAAAATGCCATGGT 720
GGCCGAGGGAACTGGCTCTACTCCACAAACAGGGGTTCGGCTTTAGGTTTTACGGTACCA
Thr Gly Ser Leu Asp Arg Asp Glu Val Phe Val Pro Lys Pro Lys Ser Lys Met Pro Trp

TTTCTAAGTACTTCAACAACCCACCAGGTAGGGTCCTCACTCTTTTGATCACACTCACTC 780
AAAGATTCATGAAGTTGTTGGGTGGTCCATCCCAGGAGTGAGAAAACTAGTGTGAGTGAG
Phe Ser Lys Tyr Phe Asn Asn Pro Pro Gly Arg Val Leu Thr Leu Leu Ile Thr Leu Thr

TAGGCTGGCCCTTGTACTTAGCCTTGAATGTTTCTGGCCGACCCTATGATCGTTTTGCTT 840
ATCCGACCGGGAACATGAATCGGAACTTACAAAGACCGGCTGGGATACTAGCAAAACGAA
Leu Gly Trp Pro Leu Tyr Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala

GCCACTATGATCCCTATGGCCCCATTTATTCCAATCGCGAAAGGTGTCAAATATTTGTCT 900
CGGTGATACTAGGGATACCGGGGTAAATAAGGTTAGCGCTTTCCACAGTTTATAAACAGA
Cys His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg Glu Arg Cys Gln Ile Phe Val

CGGATGCTGGTGTCTTTGCTACAACTTATGTGCTTTACTACGCAGCAATGTCAAAAGGGC 960
GCCTACGACCACAGAAACGATGTTGAATACACGAAATGATGCGTCGTTACAGTTTTCCCG
Ser Asp Ala Gly Val Phe Ala Thr Thr Tyr Val Leu Tyr Tyr Ala Ala Met Ser Lys Gly

TGGCATGGCTTGTATTCATTTATGGTATGCCATTGCTCATAGTGAATGGCTTCCTTGTAT 1020
ACCGTACCGAACATAAGTAAATACCATACGGTAACGAGTATCACTTACCGAAGGAACATA
Leu Ala Trp Leu Val Phe Ile Tyr Gly Met Pro Leu Leu Ile Val Asn Gly Phe Leu Val

TAATCACCTACTTGCAGCACACTCACCCTGCATTGCCGCACTATGACTCATCAGAATGGG 1080
ATTAGTGGATGAACGTCGTGTGAGTGGGACGTAACGGCGTGATACTGAGTAGTCTTACCC
Leu Ile Thr Tyr Leu Gln His Thr His Pro Ala Leu Pro His Tyr Asp Ser Ser Glu Trp

ATTGGCTTAGGGGGGCATTGGCGACGGCGGATAGAGATTACGGAATGCTGAATAAGGTTT 1140
TAACCGAATCCCCCCGTAACCGCTGCCGCCTATCTCTAATGCCTTACGACTTATTCCAAA
Asp Trp Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp Tyr Gly Met Leu Asn Lys Val

TCCACAATATCATAGACACCCATGTGGCTCACCATCTCTTCTCTACCATGCCTCATTACC 1200
AGGTGTTATAGTATCTGTGGGTACACCGAGTGGTAGAGAAGAGATGGTACGGAGTAATGG
Phe His Asn Ile Ile Asp Thr His Val Ala His His Leu Phe Ser Thr Met Pro His Tyr

ATGCAATGGAAGCCACCAAAGCAATCAAGTCAATATTGGGCAAATACTACCAGTTTGATG 1260
TACGTTACCTTCGGTGGTTTCGTTAGTTCAGTTATAACCCGTTTATGATGGTCAAACTAC
His Ala Met Glu Ala Thr Lys Ala Ile Lys Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp

GCACTCCAGTTTACAAGGCAGTGTGGAGGGAGGCTAAAGAGTGCCTTTATGTTGAGTCGG 1320
CGTGAGGTCAAATGTTCCGTCACACCTCCCTCCGATTTCTCACGGAAATACAACTCAGCC
Gly Thr Pro Val Tyr Lys Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val Glu Ser

ACGAGGGGGCCCCTAACAAAGGTGTTTTCTGGTATCAGAGCAAGCTGTGATATTGGCTGG 1380
TGCTCCCCCGGGGATTGTTTCCACAAAAGACCATAGTCTCGTTCGACACTATAACCGACC
Asp Glu Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln Ser Lys Leu

ATAGAGCCAAAGAAAATGTGATTAGTAAGGTAGTGTCTTTGGTCAGTTTGGTGTGTTAAG 1440
TATCTCGGTTTCTTTTACACTAATCATTCCATCACAGAAACCAGTCAAACCACACAATTC

GAACAAATAATAATAATTAGCGACTATGAATAGTTATTGTTAAACAAAATTCACCCTTAT 1500
CTTGTTTATTATTATTAATCGCTGATACTTATCAATAACAATTTGTTTTAAGTGGGAATA

GTTTAGCAGGAACTTTTCTGGCTACACTTTTTTTCGTATGAAAAGCGCATATTTTTTAAT 1560
CAAATCGTCCTTGAAAAGACCGATGTGAAAAAAAGCATACTTTTCGCGTATAAAAAATTA

TGTTATATTGTTTTGACATTACTCAAGCTTCAAAATTAATATCACAGAAAATATCCAATG 1620
ACAATATAACAAAACTGTAATGAGTTCGAAGTTTTAATTATAGTGTCTTTTATAGGTTAC

TCGAAGGTTTCATTGTAGGTTGAAAACTTTATATTGAGGTGG 1662
AGCTTCCAAAGTAACATCCAACTTTTGAAATATAACTCCACC

```
         10        20        30        40        50
tccaacgctctctcctacgctcgttctcatatgttgtttatgccctctcc 50
ttagccttcctcttctactatattgctacctcttccttccatctcctccc 100
tcaccccctttcctacttggcatggtcaatctattgggctctcccaggct 150
gcattctcccggcgtttgggtcctcgcacctgcgtgcggtcacccatgcc 200
tttagtgcctcctcatgggttgatgcccatggttggcctcccccttccctc 250
tgctcttttagttcccatacttttcatggacgcttcgccctgtcgccccc 300
cctctccccccggctcccttgaccgagctgcggtgtttgtccccccgccg 350
acctcccacctgccctggttttctacgtacttcaaccaccccccaggtcg 400
ggtcctccctcttttgatccccctcactctcggctggcccttgtccttcg 450
ccttgcctgtttctggccgcccctatgatcgttttgcttgcccctctgct 500
ccctatggcccatttcattcccctcgcgaccggtgtcccctatttgtctc 550
ggatgctggtgtctttgctaccccttatgtgctttactccgcagcccatgt 600
caccagggctggcctggcttgtcttcatttctggtctgcccttgctcctc 650
gtgcctggcttccttgtcttactcccctccttgccgcccccctcccccctgc 700
cttgccgcccctatgcctcctccgcctgggcttggcttcgggggggcattgg 750
cgccggcggctcgcgattccggcctgctgcctccggttttcccccctctc 800
atagccccccatgtggctcccccctctcttctctcccctgcctcattcccc 850
tgccctggacgcccccccagcaatccagtccctcttgggccccctcctccc 900
cgtttgctggcactcccgtttcccggccgtgtggcgggcggctcccgag 950
tgcctttctgttgcgtcggccgcgggggcccctcccccggtgttttctg 1000
gtatccgcgcccgctgtgctattggctggctcgcgcccccgcccatgtgc 1050
ttcgtccggtcgtgtctttggtccgtttggtgtgttccggcccccctcct 1100
cctccttcgcgcctatgcatcgttattgttccc 1133
```

Fig. 3 - Nucleotide sequence of cDNA clone "I".

Fig 4 - Nucleotide sequence alignment of clones "I" (I.SEQ) and "N2" (N2.SEQ).

```
1    ---------------------------------------- I.SEQ
1    CCTCATAAAAAAGTAAGCTCATTTACCTCAAGTAGGGTTT N2.SEQ

1    ---------------------------------------- I.SEQ
41   CCTTATGACAAATGAGTCCCGCAATCCTTTTCTATGAGGT N2.SEQ

1    ---------------------------------------- I.SEQ
81   GCTATAATTGCAAATGTCCAAATCATAGGGATATGGATCC N2.SEQ

1    ---------------------------------------- I.SEQ
121  AAATACTATTAATATTATGTAGTGTGTTTTTTTTTTTCCC N2.SEQ

1    ---------------------------------------- I.SEQ
161  TCAAATTTACTCTCACACCTAAGTTGATTTTCTCCAGCAT N2.SEQ

1    ---------------------------------------- I.SEQ
201  TGGACATAGCCTCTGTAGACAATGGGAGCTAGAAGCCGAA N2.SEQ

1    ---------------------------------------- I.SEQ
241  TGCCTGCTACCAACAAGCCTAAAGAGCAAAAAACACCCAT N2.SEQ

1    ---------------------------------------- I.SEQ
281  CCAGCGAGCACCACACACAAAACCCCCATTCACTCTTAGC N2.SEQ

1    -------------------------------TCCAACGCT I.SEQ
321  CAACTCAAGAAAGCCGTCCCACCCAATTGTTTCCAACGCT N2.SEQ

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361  CTCTCCTACGCTCGTTCTCATATGTTGTTTATGACCTCTC N2.SEQ

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401  CTTAGCCTTCCTCTTCTACTATATTGCTACCTCTTACTTC N2.SEQ

90   CATCTCCTCCCTCACCCCCTTTCCTACTTGGCATGGTCAA I.SEQ
441  CATCTCCTCCCTCACCCCCTTTCCTACTTGGCATGGTCAA N2.SEQ

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170  GGTCATCGCACATGAGTGCGGTCACCATGCCTTTAGTGAC I.SEQ
521  GGTCATCGCACATGAGTGCGGTCACCATGCCTTTAGTGAC N2.SEQ

210  TACCAATGGGTTGATGACATGGTTGGCCTAACCCTTCACT I.SEQ
561  TACCAATGGGTTGATGACATGGTTGGCCTAACCCTTCACT N2.SEQ

250  CTGCTCTTTTAGTTCCATACTTTTCATGGAAGATTAGCCA I.SEQ
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290  CTGTCCCCACCACTCTAACACCGGCTCCCTTGACCGAGAT I.SEQ
641  CTGTCGCCACCACTCTAACACCGGCTCCCTTGACCGAGAT N2.SEQ

330  GAGGTGTTTGTCCCCAAGCCGAAATCCAAAATGCCATGGT I.SEQ
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370  TTTCTAAGTACTTCAACAACCCACCAGGTAGGGTCCTCAC I.SEQ
721  TTTCTAAGTACTTCAACAACCCACCAGGTAGGGTCCTCAC N2.SEQ

410  TCTTTTGATCACACTCACTCTAGGCTGGCCCTTGTACTTA I.SEQ
761  TCTTTTGATCACACTCACTCTAGGCTGGCCCTTGTACTTA N2.SEQ

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530 A A G G T G T C A A A T A T T T G T C T C G G A T G C T G G T G T C T T T G C T I.SEQ
881 A A G G T G T C A A A T A T T T G T C T C G G A T G C T G G T G T C T T T G C T N2.SEQ

570 A C A A C T T A T G T G C T T T A C T A C G C A G C A A T G T C A A A A G G G C I.SEQ
921 A C A A C T T A T G T G C T T T A C T A C G C A G C A A T G T C A A A A G G G C N2.SEQ

610 T G G C A T G G C T T G T A T T C A T T T A T G G T A T G C C A T T G C T C A T I.SEQ
961 T G G C A T G G C T T G T A T T C A T T T A T G G T A T G C C A T T G C T C A T N2.SEQ

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1   M G A G G R T D V P P A N R K S E V D P L K R V P F E K P Q F S L S Q I K K A I   L43921.PRO
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360 E C L Y V E S D E G A P N K G V F W Y Q S K L                                       N2.PRO
361 E C I Y V E P D Q S T E S K G V F W Y N N K L                                       L43921.PRO
361 E C I Y V E P D R E G D K K G V Y W Y N N K L                                       L26296.PRO
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Fig. 5 - Aminoacid sequence alignment of Δ12 desaturase from hazelnut (N2.PRO), *Arabidopsis* (L26296.PRO) and soybean (L43921.PRO). Homologous residues are boxed.

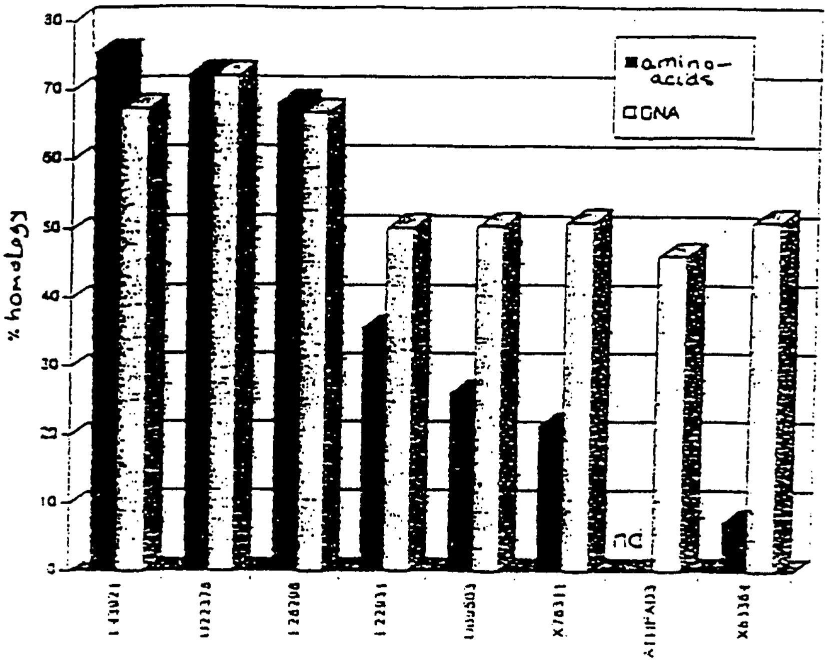

Fig. 6 – Homology between hazel Δ12 desaturase and other desaturases

L43921: Δ12 desaturase of the endoplasmic reticulum of soya
U22378: Δ12 hydroxylase of ricin
L26296: Δ12 desaturase of the endoplasmic reticulum of *Arabidopsis thaliana*
L22931: Δ15 plastid desaturase of *Arabidopsis thaliana*
U09503: Δ12 plastid desaturase of *Arabidopsis thaliana*
X78311: Δ12 plastid desaturase of spinach
ATHFAD3: Δ15 desaturase of the endoplasmic reticulum of *Arabidopsis thaliana*
X63364: Δ9 plastid desaturase of rape

Note: nd: not determined since the amino-acid sequence is not known.

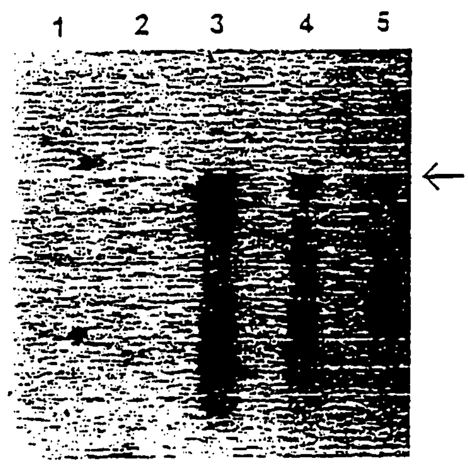

Fig. 7 - Northern blot of RNA of Montebello leaves (line 1), Nocchione leaves (line 2), Montebello kernels (line 3), Nocchione kernels (line 4), and San Giovanni kernels (line 5). The RNA was hybridized with the I clone of cDNA.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 10 3098

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WO 94 11516 A (DU PONT ;LIGHTNER JONATHAN EDWARD (US); OKULEY JOHN JOSEPH (US)) 26 May 1994 examples 1,6,7 ---	10,13	C12N15/53 C12N15/82 C12N9/02 C12N5/10 C12Q1/68 //A01H5/00
A,D	THE PLANT CELL, vol. 6, January 1994, pages 147-158, XP002034147 OKULEY, J., ET AL . : "ARABIDOPSIS FAD2 GENE ENCODES THE ENZYME THAT IS ESSENTIAL FOR POLYSATURATED LIPID SYNTHESIS" * page 155, column 2, line 28 * ---	1-14	
A	WO 95 22598 A (DU PONT ;LETO KENNETH JOSEPH (US); ULRICH JAMES FRANCIS (US)) 24 August 1995 * page 10, line 1 * -----	1-23	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	3 July 1997	Holtorf, S

CATEGORY OF CITED DOCUMENTS

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